Supplementary Information

A Humanized Ossicle-niche Xenotransplantation Model Enables Improved Human Leukemic Engraftment

Andreas Reinisch, Daniel Thomas, M. Ryan Corces, Xiaohua Zhang, Dita Gratzinger, Wan-Jen Hong, Katharina Schallmoser, Dirk Strunk, and Ravindra Majeti

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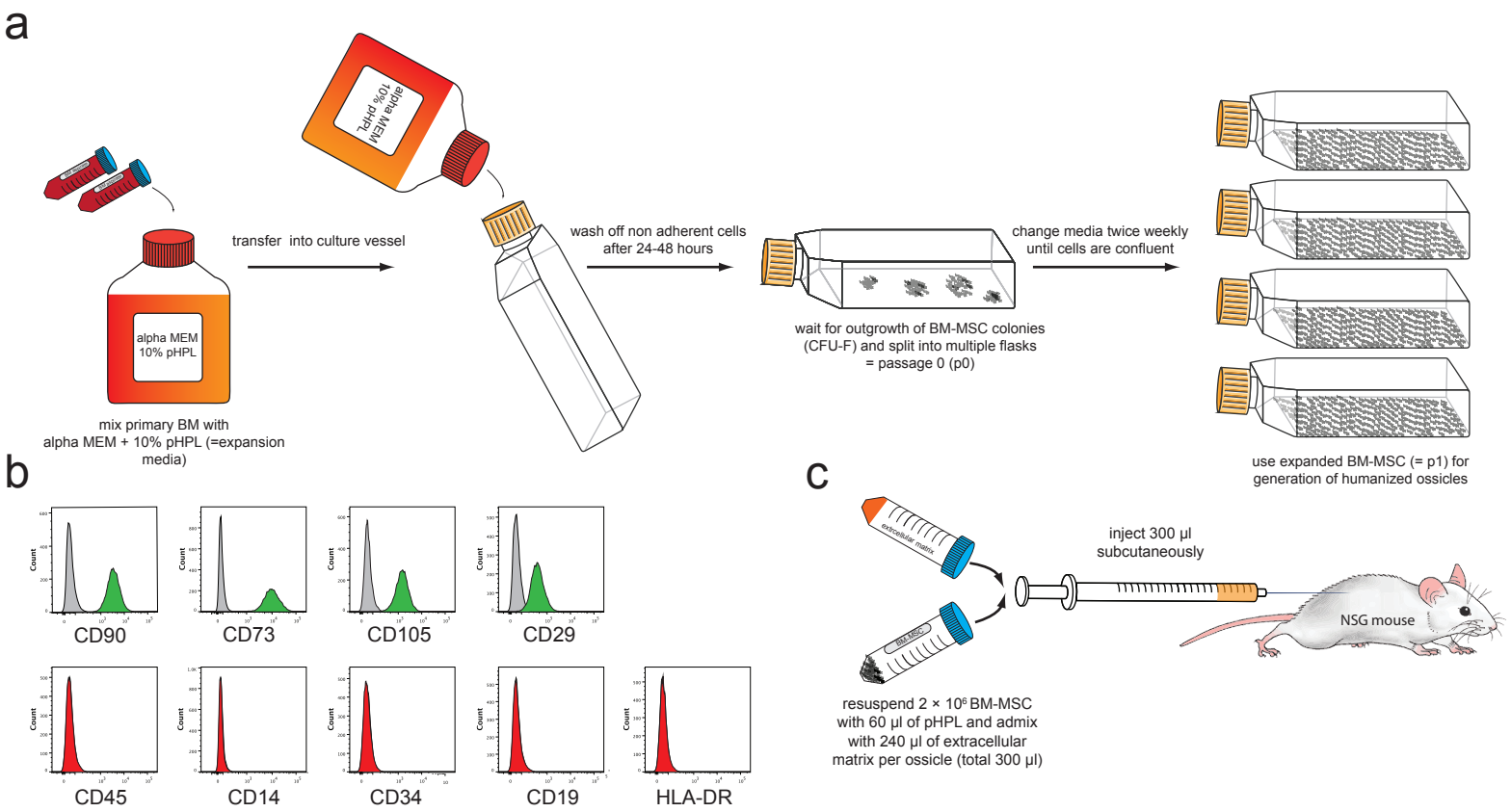
Supplementary Figure 1



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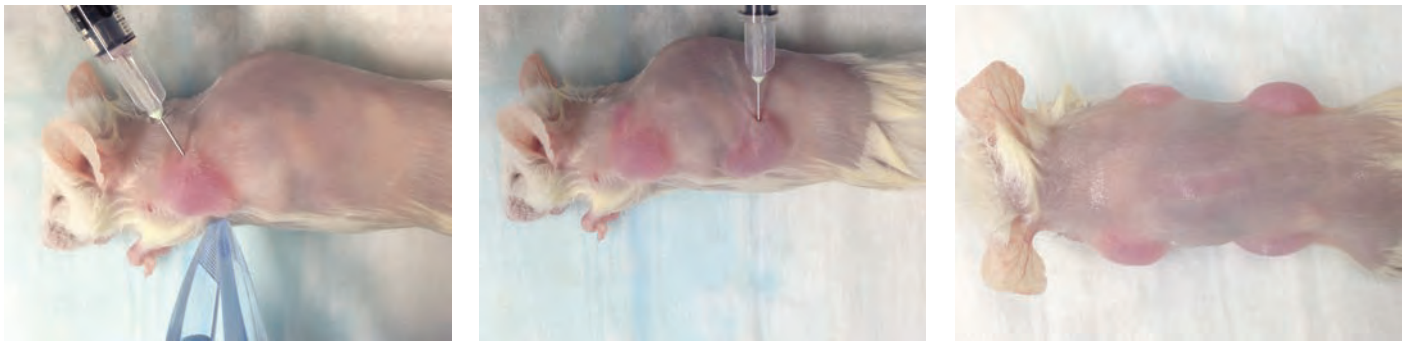

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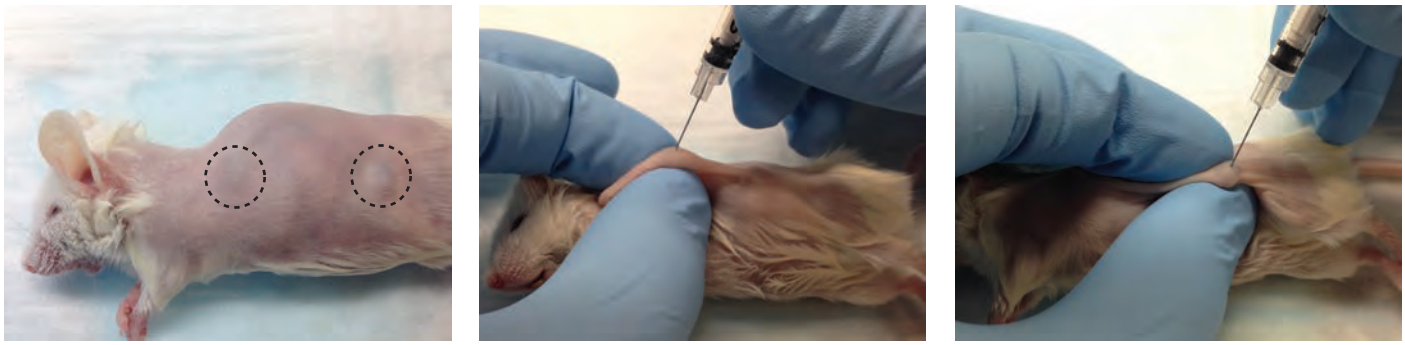

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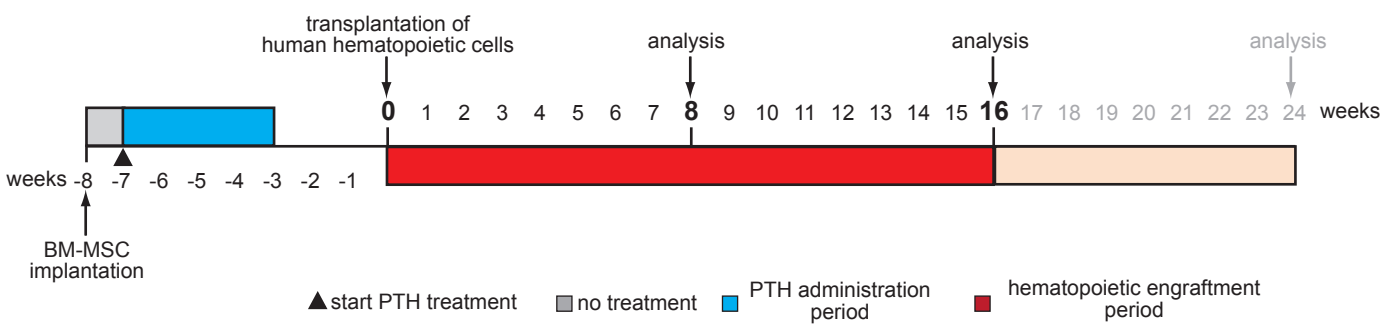

Supplementary Figure 1

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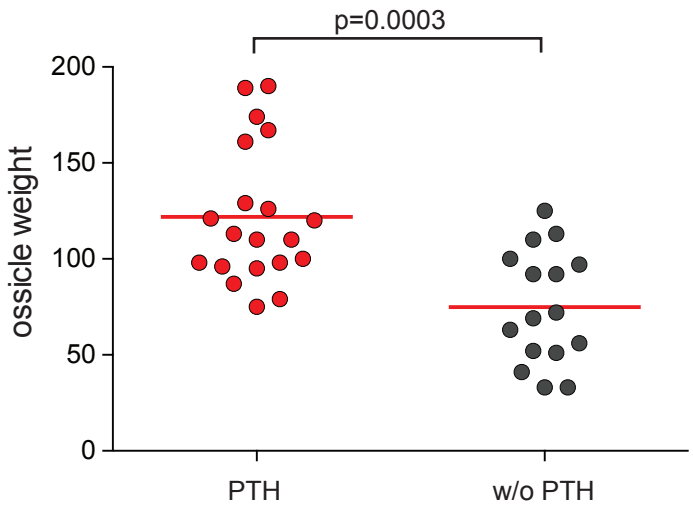

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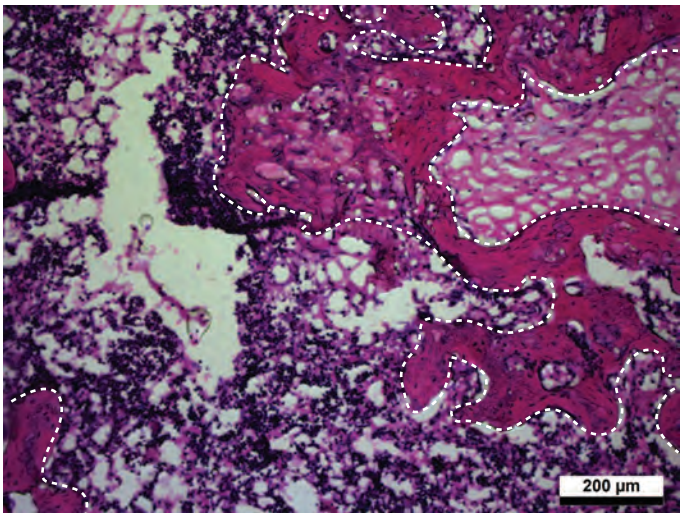

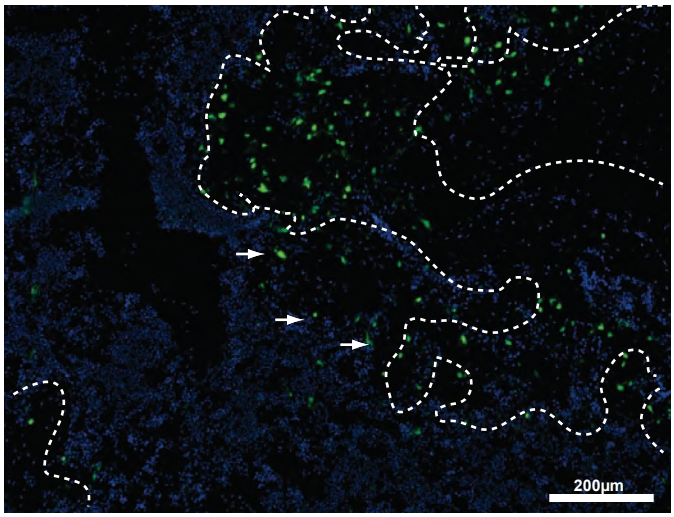

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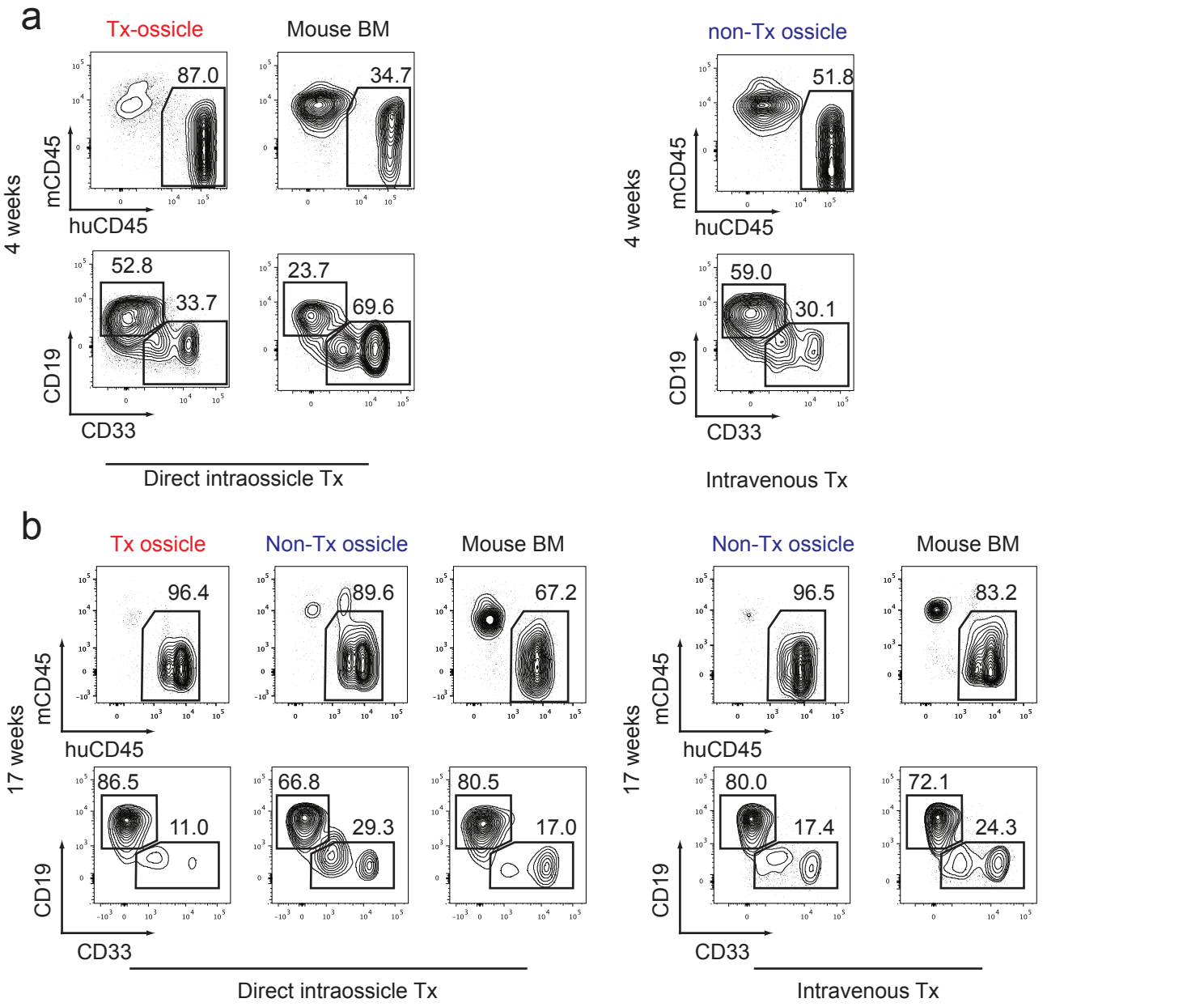

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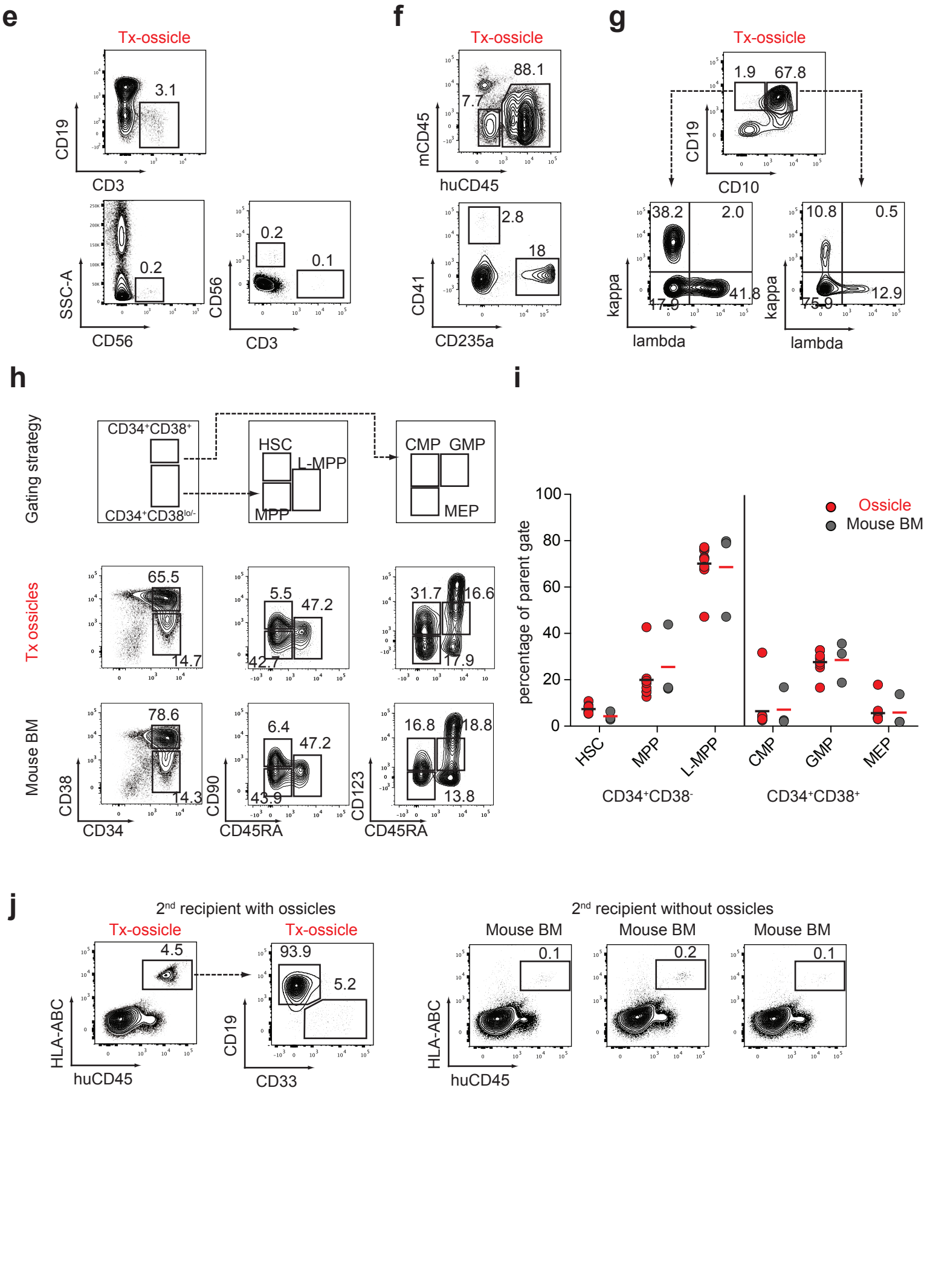

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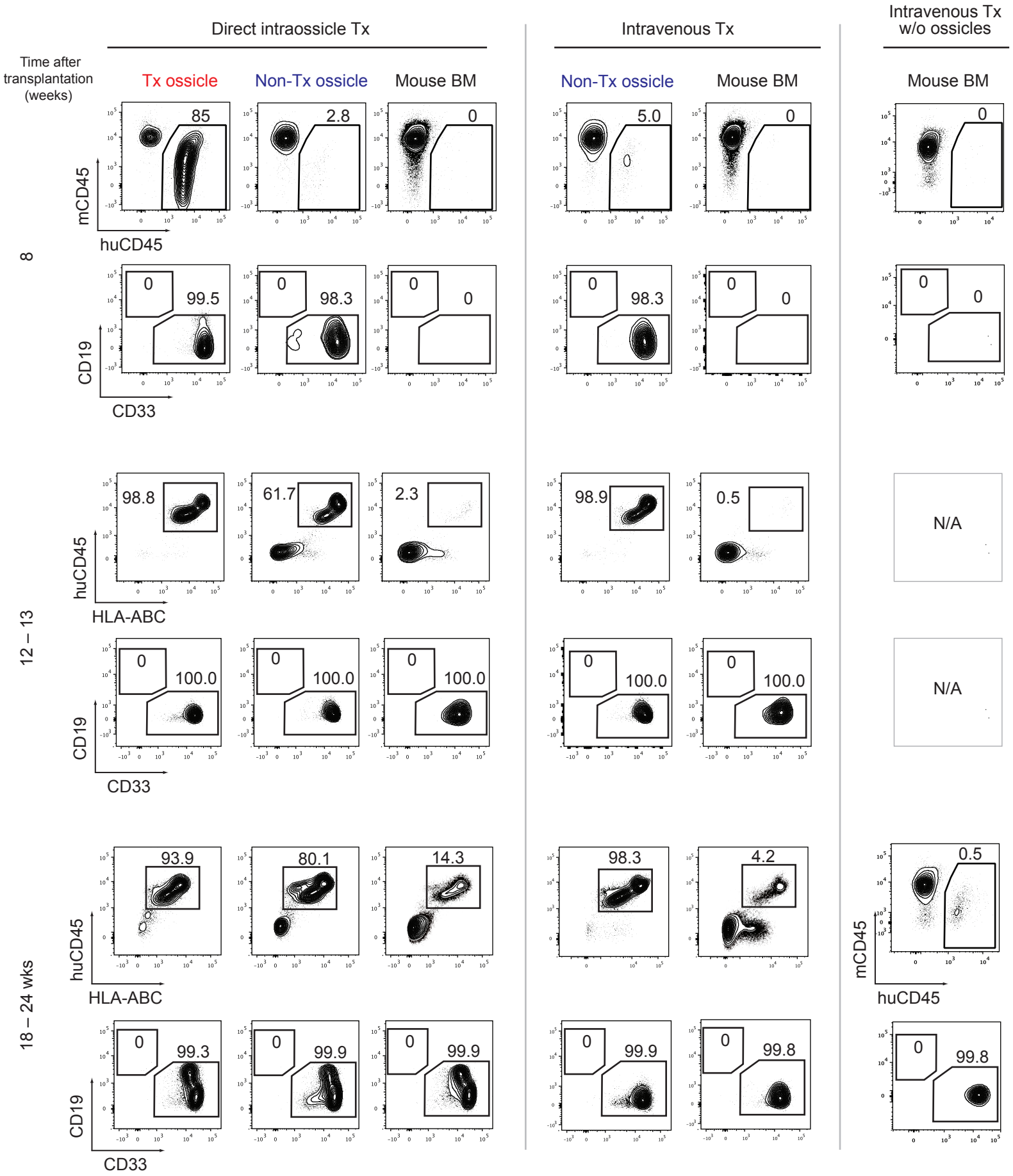

Supplementary Figure 4

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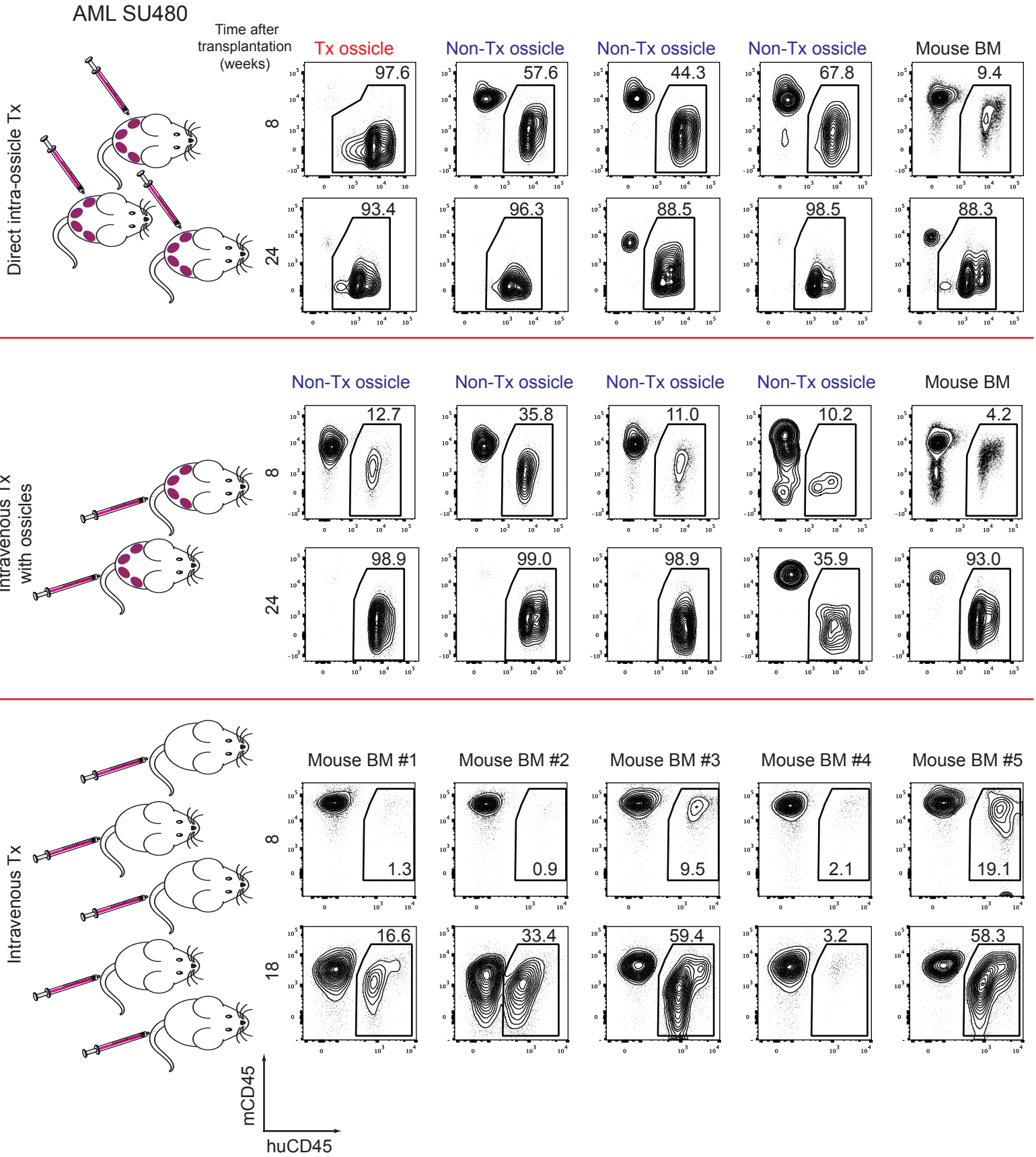

Supplementary Figure 4

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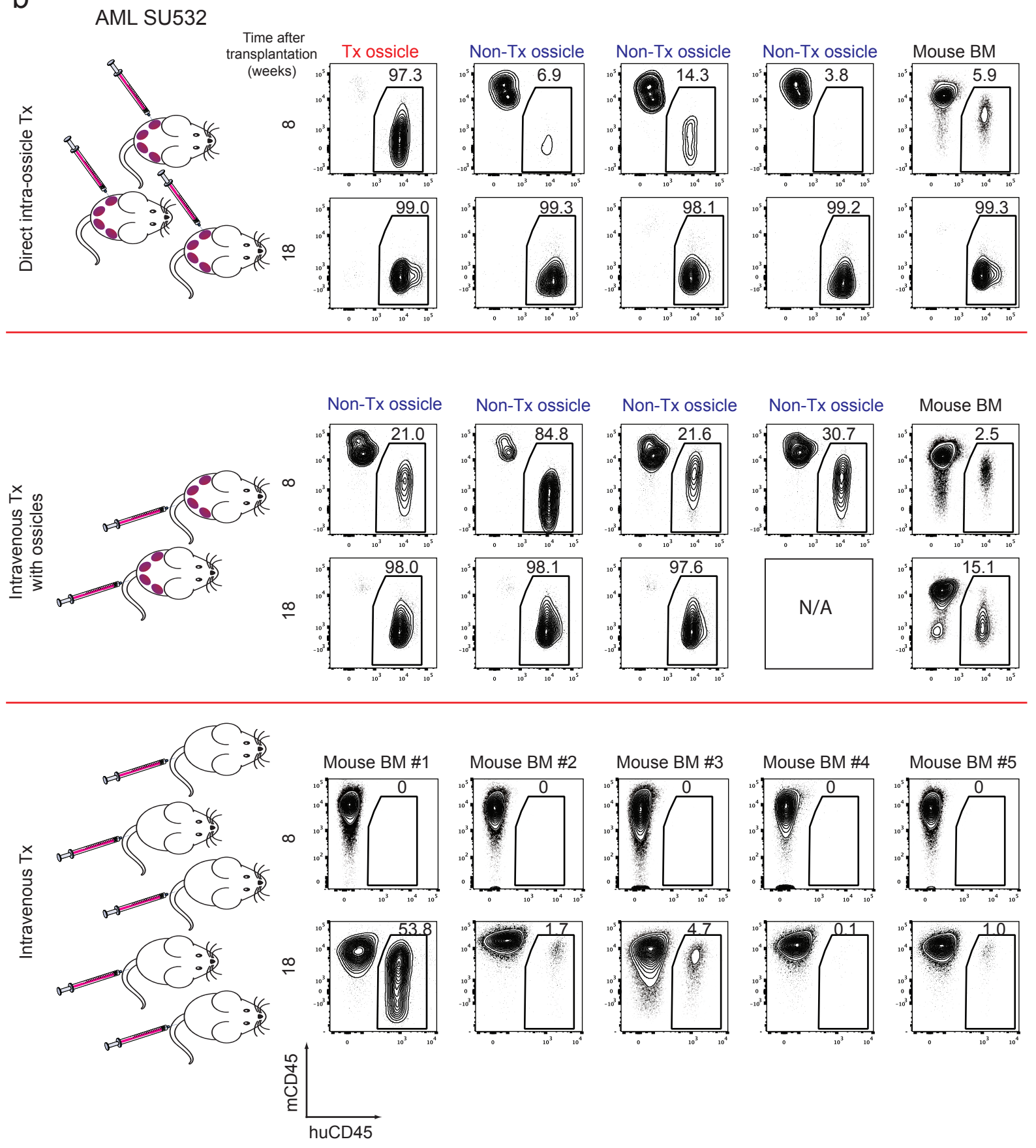

Supplementary Figure 4

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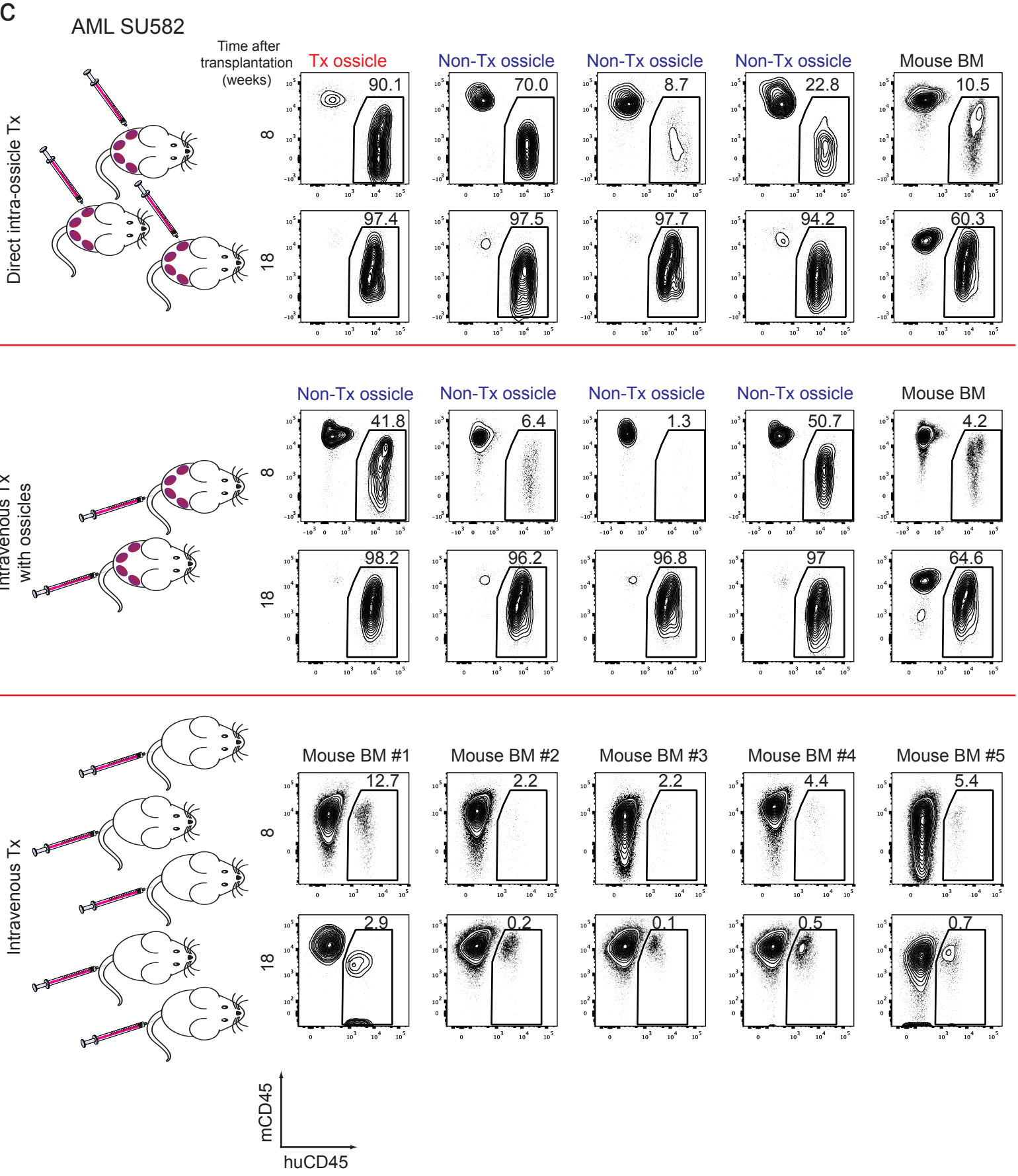
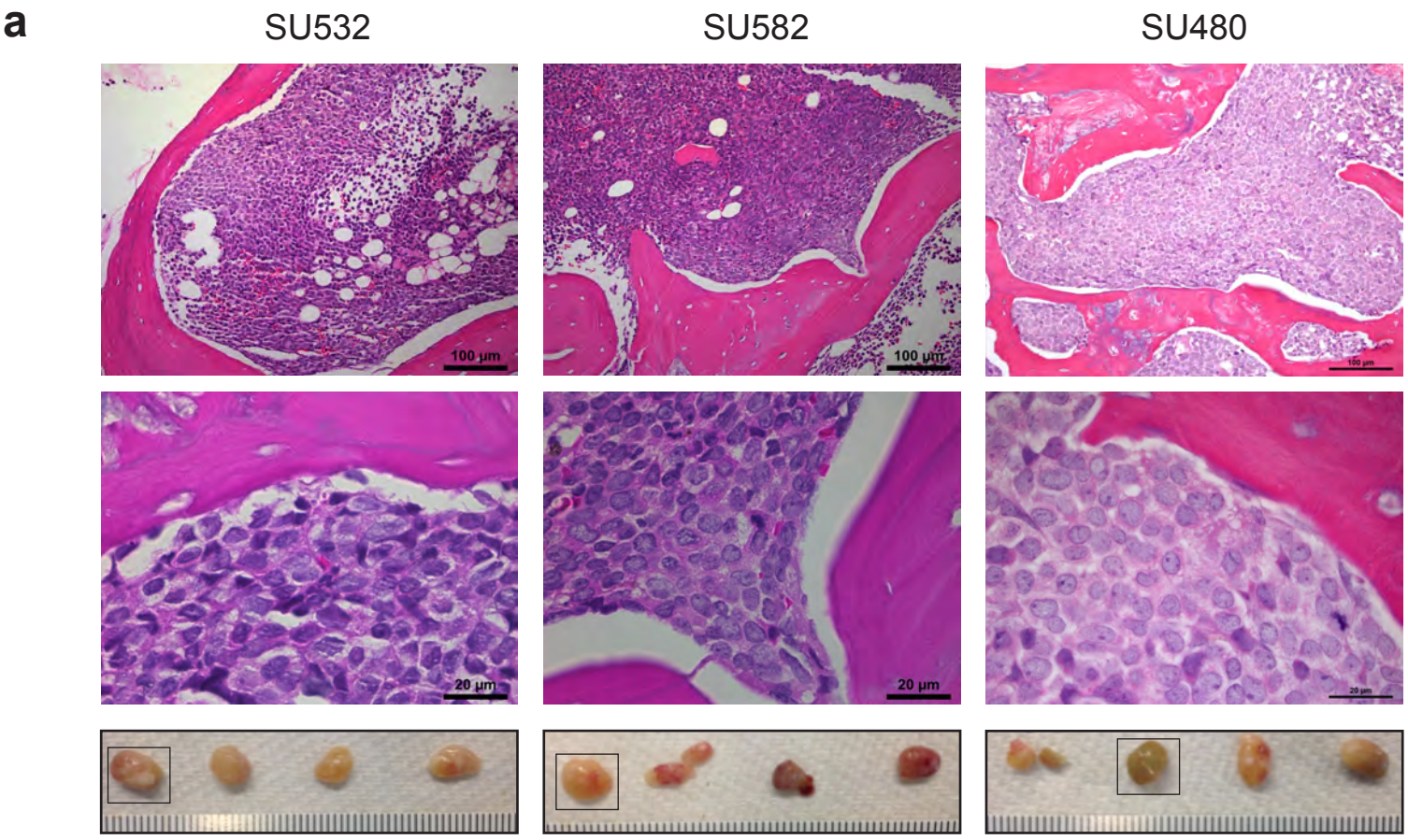
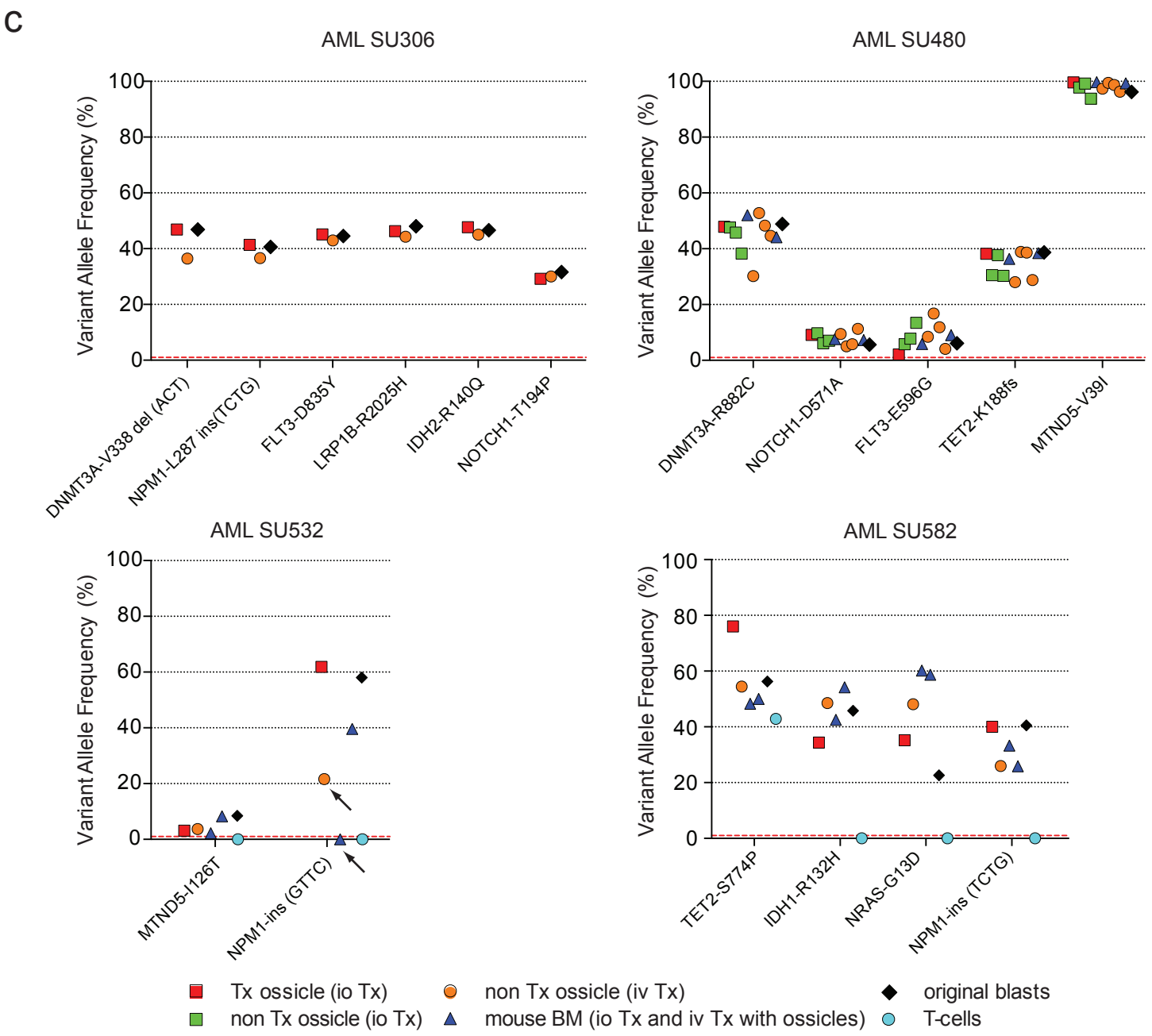
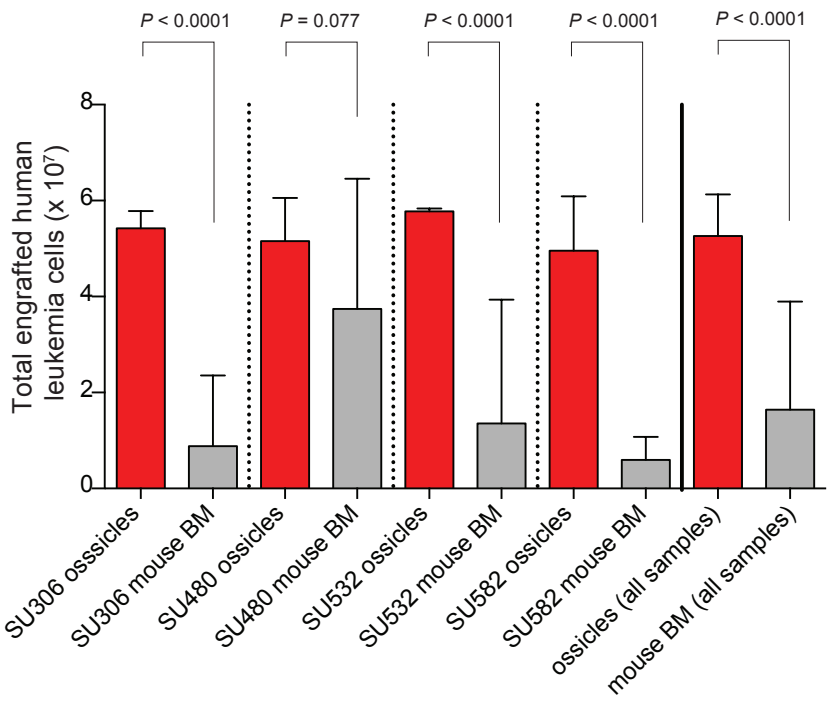


Supplementary Figure 5



Supplementary Figure 5





Supplementary Figure 6

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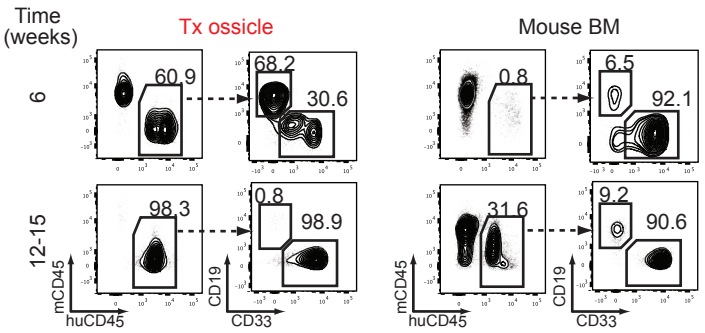

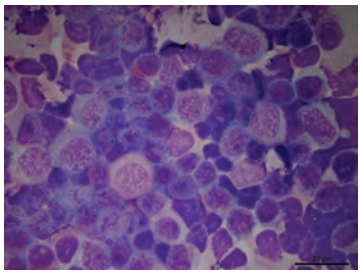

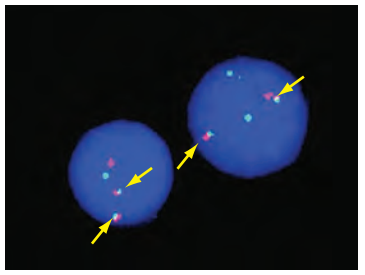

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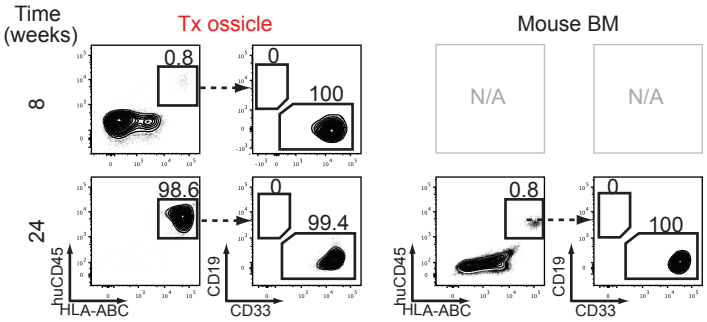

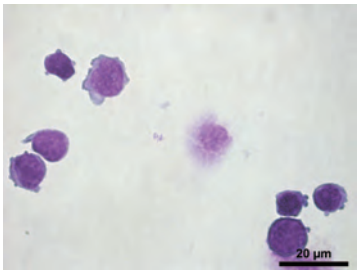

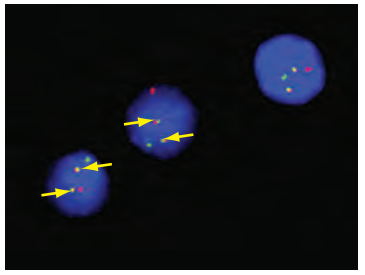

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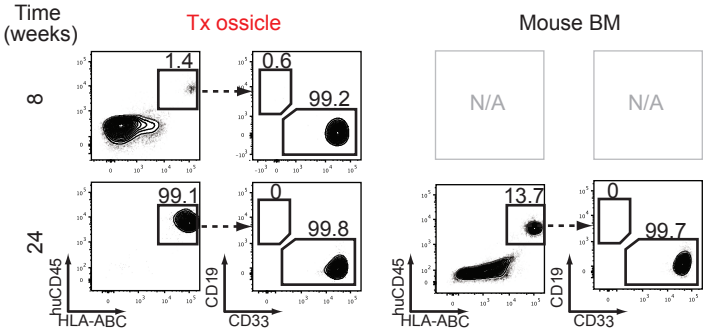

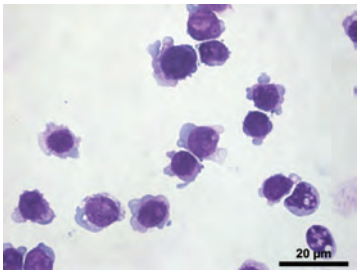

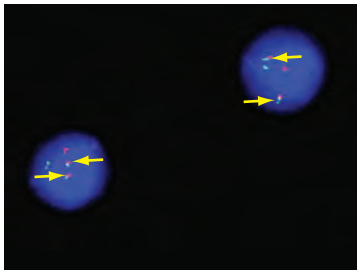

Supplementary Figure 6

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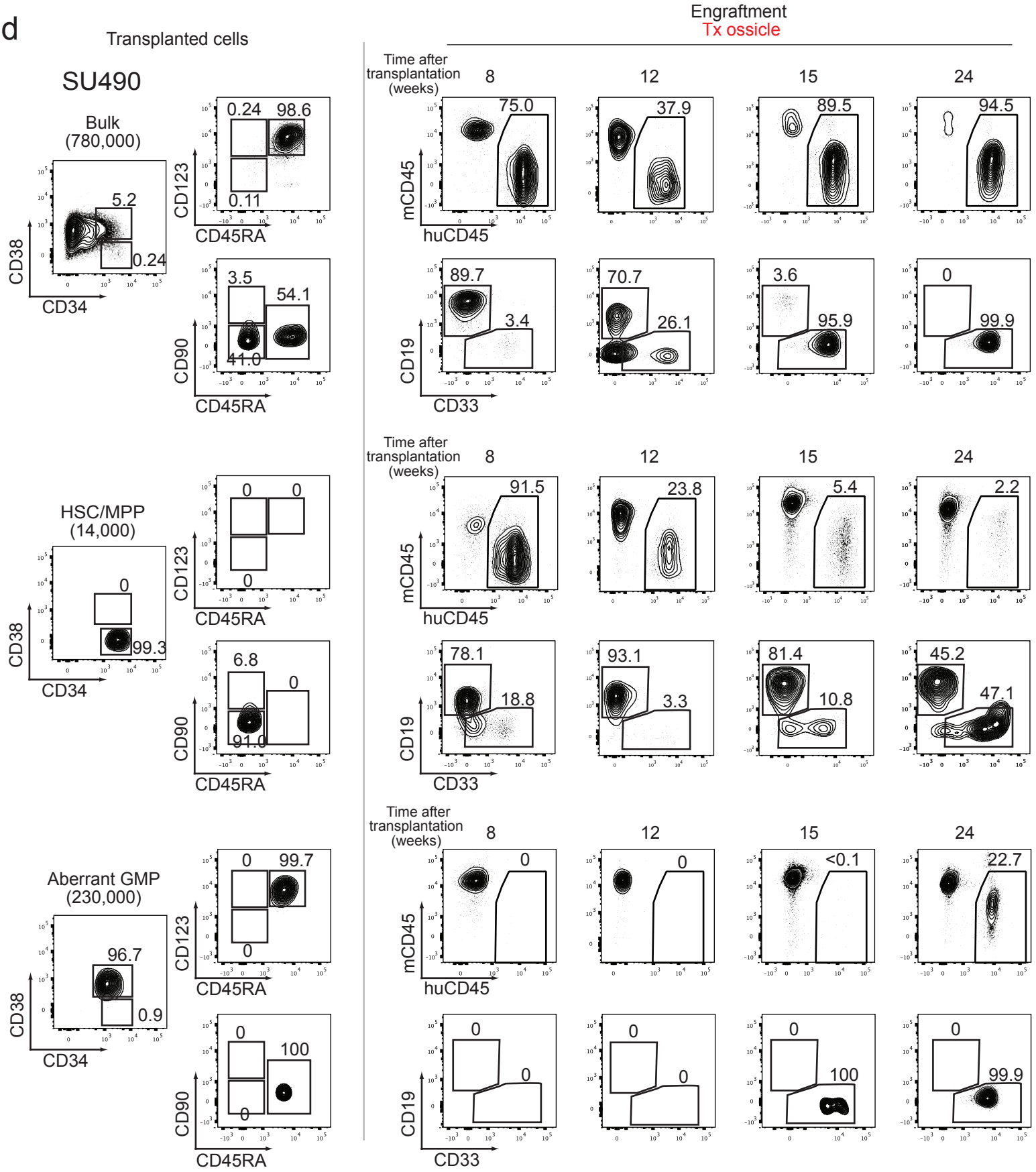

e

sample ID	transplanted cells	FACS sorted fraction analyzed	No. of nuclei analyzed	results	comments
SU490	HSC/MPP	CD19+	200	negative	no cells with fusion observed
		CD33+	200	negative	no cells with fusion observed
	bulk	CD33+	200	negative	0.5% with PML/RARA fusion (1/200)
	aberrant GMP	CD33+	156	positive	98% with PML/RARA fusion (153/156)
SU748	HSC/MPP	CD19+	200	negative	no cells with fusion observed
		CD33+	200	negative	no cells with fusion observed
	bulk	CD33+	200	positive	98.5% with PML/RARA fusion (197/200)

Supplementary Figure 7

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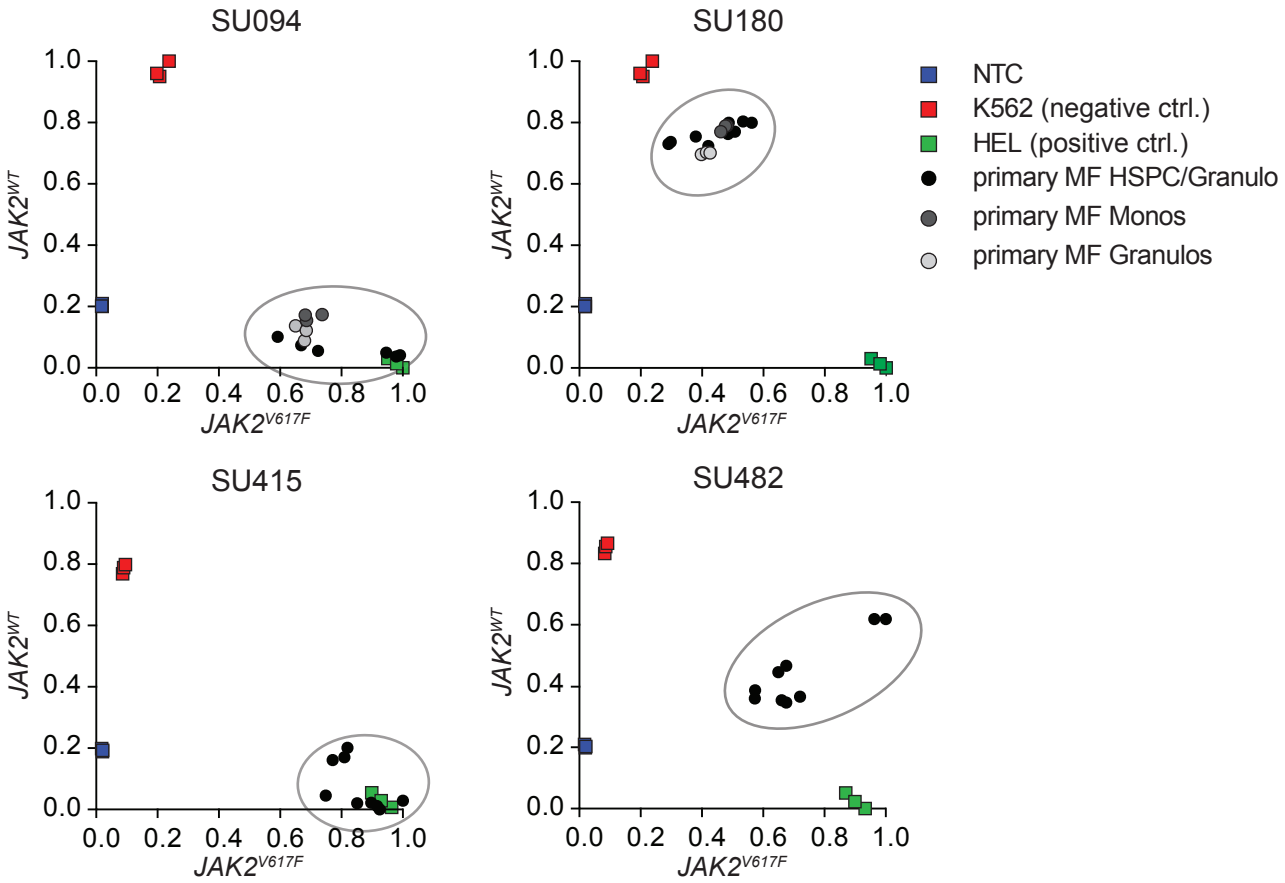

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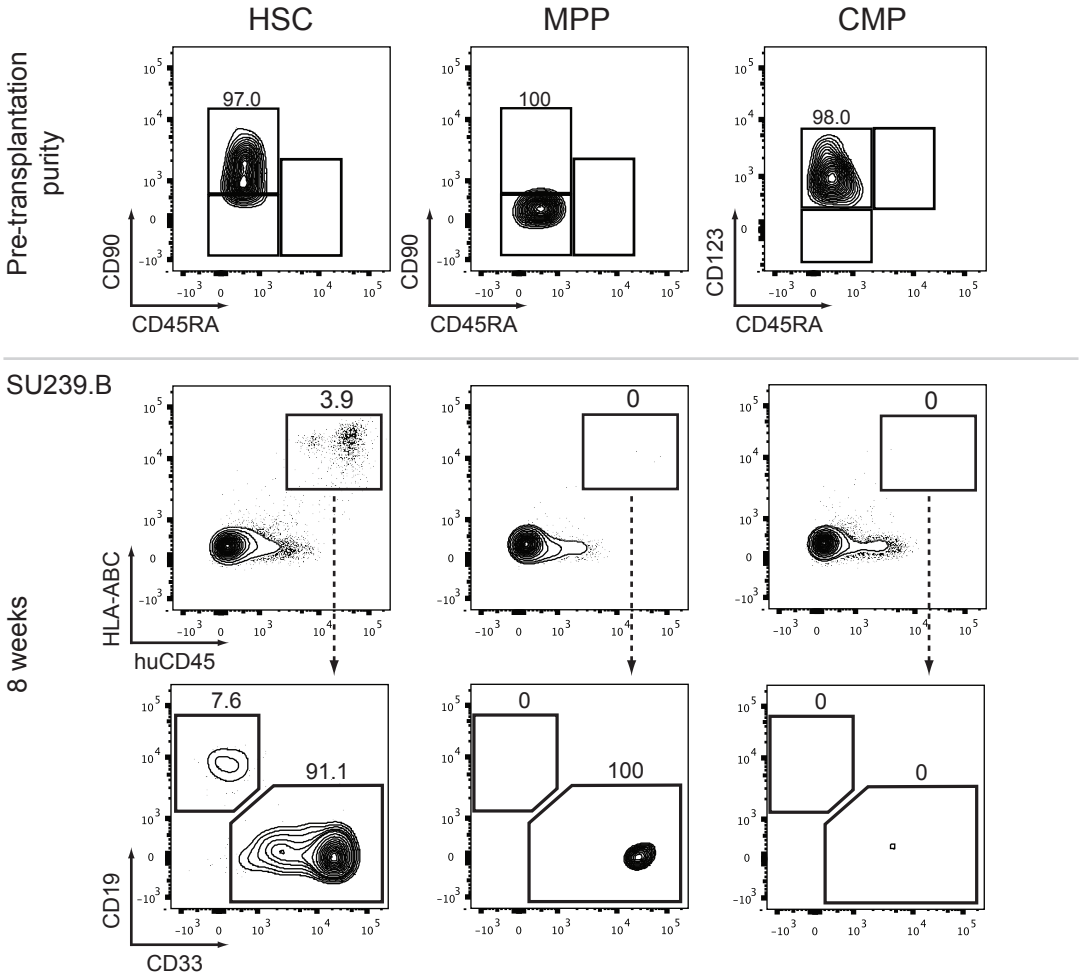

Supplementary Figure 7

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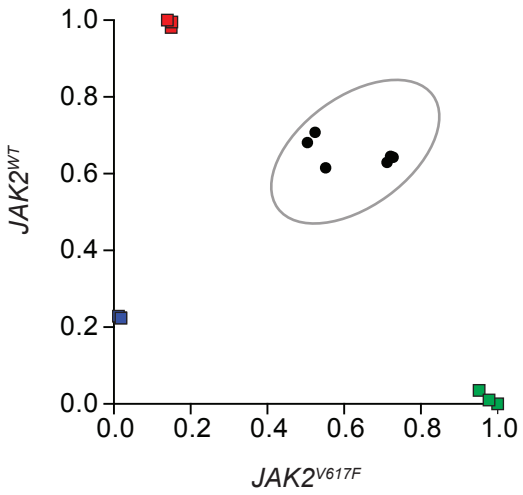
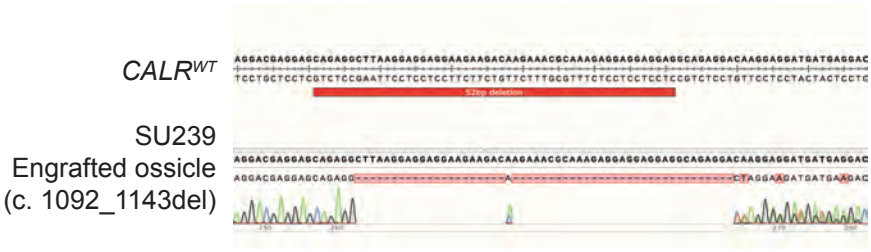


NTC
K562 (negative ctrl.)
HEL (positive ctrl.)
SU482 HSC Tx, ossicle-derived myeloid cells

d



SUPPLEMENTAL FIGURES AND TABLES

Supplementary Figure 1. BM-MSC isolation, propagation, and humanized ossicle niche formation. (**a**) Experimental schematic describing isolation and propagation of human BM-derived MSC. Human total BM cells (from fresh BM aspirates or bag washouts) were admixed with expansion medium consisting of alpha modified minimum essential medium (alpha MEM) supplemented with 10% pooled human platelet lysate (pHPL)[1,2] and seeded into cell culture vessels. After 24 to 48 hours non-adherent cells were removed by rinsing with pre-warmed PBS and the adherent cells were further expanded until the outgrowth of colonies (colony unit forming fibroblasts, CFU-F) was observed. These cells are considered passage 0 (p0). All p0 cells were detached from the plastic and sub-passaged into multiple flasks or cell factories to provide enough culture surface area to allow for sufficient expansion. Expanded cells (passage 1) were frozen or immediately used for the generation of humanized ossicle niches. (**b**) Purity of isolated MSC was analyzed by flow cytometry using a consensus panel of positive and negative markers.[3] Histograms show cell surface staining for CD90, CD73, CD105, and CD29 (green) compared to isotype control (grey). BM-MSC did not express hematopoietic markers CD45, CD14, CD34, CD19, and HLA-DR (red). (**c**) Experimental schematic describing subcutaneous BM-MSC transplantation. 2×10^6 BM-MSC were re-suspended in 60 µl of pHPL and admixed with 240 µl of extracellular matrix (Angiogenesis assay kit, Millipore) prior to subcutaneous injection (total volume: 300 µl per injection) into the flanks of 6 – 8 week old female NSG mice. (**d**) Images showing subcutaneous injection of cell-matrix mixtures into the left front (left image) and left back (middle image) flank of a shaved NSG mouse. Up to four injections were done per mouse (right image). (**e**) Images showing humanized ossicle-niches formed eight weeks after MSC transplantation visible through the skin of shaved mice. (left image). Dashed circles highlight a purple hue indicative of proper ossicle niche formation and murine hematopoietic engraftment. Humanized ossicle-niches are readily accessible for direct intra-ossicle transplantation and aspiration of human hematopoietic cells (middle and right image). (**f**)

Experimental timeline. Starting at 3 – 7 days after BM-MSC transplantation (week -7) daily treatment of anabolic doses (40 µg/kg BW) of human 1-34 parathyroid hormone (PTH) is carried out for 28 consecutive days. Ossicle formation is evaluated visually (color change to purple indicates hematopoietic engraftment and BM niche formation, see dashed lines f, left image) weekly starting at week -2. Human hematopoietic cells (HSPC, AML, PMF) are transplanted and engraftment is analyzed subsequently at indicated time-points up to 24 weeks post transplantation. (**g**) Anabolic PTH treatment increases size of humanized ossicle niches. Mice were either treated with anabolic doses (40 µg/kgBW) of 1-34 PTH for 28 consecutive days starting 3 – 7 days after BM-MSC transplantation (red dots) or no treatment was applied (grey dots). Weight of individual ossicles was evaluated after excision. Each dot represents one ossicle, lines represent mean. (n = 20 for PTH treatment , n = 16 for w/o PTH, P = 0.0003, t-test) (**h**) GFP-transduced human BM-MSC form humanized ossicle niches. Microscopic images showing H/E (left) and fluorescence (right) of explanted ossicles generated from 2 × 10^6 lentivirally-transduced cells expressing GFP. GFP^+ human MSC-derived cells reside within bone structures (outline of bone area is marked by dotted white lines) and within the hematopoietic tissue of the marrow space (white arrowheads). Nuclei are stained with DAPI. Scale bar: 200 µm

Supplementary Figure 2. Human hematopoietic stem and progenitor cells engraft humanized ossicle niches. (**a,b**) FACS analysis showing human hematopoietic engraftment at four and 17 weeks post transplantation of 1 × 10^4 UCB-derived $CD34^+$ hematopoietic stem and progenitor cells (HSPC). Cells were applied either by direct intraossicle injection into one out of four ossicles or intravenously into mice with or without ossicles. Transplanted (Tx ossicle) and non-transplanted ossicles (Non-Tx ossicle), as wells as mouse bone marrow (BM) were analyzed by flow cytometry for the presence of human $CD45^+$ hematopoietic cells, further gated for myeloid cells ($CD45^+CD33^+$) and B lymphoid cells ($CD45^+CD19^+$). (**c**) FACS analysis of engrafted myeloid cells: SSC-$A^{high}CD15^+$ neutrophils, SSC-$A^{high}CD45^{high}$ eosinophils,

$CD14^{high}HLA\text{-}DR^{+}$ mature monocytes, $CD117^{+}CD11b^{lo}$ mast cells, and conventional and plasmacytoid dendritic cells (cDCs, pDCs, left to right). Parental gate for all depicted plots is $CD45^{+}HLA\text{-}ABC^{+}$. Numbers represent % of gated cells. (**d**) May-Gruenwald Giemsa stain of myeloid cells isolated by cell sorting (gating shown in (**c**)**,** same sequence of cells from left to right as in (**c**)). Scale bar, 20 µm. (**e,f**) FACS plots of (**e**) $CD3^{+}$ T-cells (upper and lower panel show 2 independent donors), $CD56^{+}$ NK-cells, (**f**) $CD41^{+}$ megakaryocyte lineage cells, as well as $CD235a^{+}$ erythroid progenitor cells (both antibodies specifically recognize human but not mouse antigens), and (**g**) mature, as well as immature, B-cells with kappa and lambda light chain expression that developed within transplanted humanized ossicle niches. Megakaryocytes were observed in histological sections, but based on H/E staining we could not distinguish if they were human- or mouse-derived. (**h**) Gating strategy (upper panel) and FACS-analysis of HSPC subsets within humanized ossicle niches (middle panel) and corresponding mouse BM (lower panel). Cells were enriched for human $CD34^{+}$ prior to analysis. $CD34^{+}$ cells are subdivided based on CD38-expression. $CD34^{+}CD38^{lo/-}$ cells are further stratified based on CD90 and CD45RA expression into hematopoietic stem cells (HSC, $CD90^{+}CD45RA^{-}$), multipotent progenitors (MPP, $CD90^{-}CD45RA^{-}$), and lymphoid-primed multipotent progenitors (L-MPP, $CD90^{-}CD45RA^{+}$), whereas $CD34^{+}CD38^{+}$ cells are fractionated into common myeloid progenitors (CMP, $CD123^{-}CD45RA^{+}$), granulocyte macrophage progenitors (GMP, $CD123^{+}CD45RA^{+}$), and megakaryocyte erythroid progenitors (MEP, $CD123^{-}CD45RA^{-}$) based on CD123 and CD45RA expression. (**i**) Engraftment summary within ossicles and the corresponding mouse BM. (**j**) Secondary engraftment of human CD34+ HSPC. 7,500 sorted CD34+ cells isolated from engrafted ossicles were transplanted into irradiated recipient mice with (left) or without ossicles (right). Engraftment in ossicles (left) and mouse bone marrow (right) 12 weeks post transplantation is indicated.

Supplementary Figure 3. All engrafted cells from primary AML SU306 express myeloid

marker CD33. FACS analysis showing human leukemia engraftment at 8, 12 – 13, and 18 – 24 weeks post-transplantation. 1 × 10^6 T cell-depleted MNCs were transplanted (i) directly into one out of four humanized ossicle niches per mouse (direct intraossicle Tx), (ii) intravenously into mice bearing four ossicles, or (iii) intravenously into mice without ossicles (top to bottom). Transplanted (Tx ossicle) and non-transplanted ossicles (Non-Tx ossicle), as wells as mouse bone marrow (BM) were analyzed for the presence of human $CD45^+HLA\text{-}ABC^+$ AML cells (week 8 engrafted cells were not stained for HLA-ABC). To confirm myeloid leukemia origin of engraftment, human cells were co-stained with CD33 and CD19. (N/A not analyzed).

Supplementary Figure 4. Primary AML SU480, SU532, and SU582 preferentially engrafts humanized ossicle niches compared to mouse bone marrow. (**a-c**) FACS analysis showing human leukemia engraftment at eight and 18 – 24 weeks post-transplantation of primary patients samples SU480 (**a**), SU532 (**b**) and SU582 (**c**). 1 × 10^6 T cell-depleted MNCs were transplanted (i) directly into one out of four humanized ossicle niches per mouse (direct intraossicle Tx), (ii) intravenously into mice bearing four ossicles, or (iii) intravenously into mice without ossicles (top to bottom). Transplanted (Tx ossicle) and non-transplanted ossicles (Non-Tx ossicle), as wells as mouse bone marrow (BM) were analyzed for the presence of human $CD45^+CD33^+$ AML cells.

Supplementary Figure 5. Analysis of primary human AML engrafted in ossicle marrow cavity. (**a**) Histological analysis (H/E stained) and gross morphology (small images below) of explanted ossicle niches fully engrafted with primary AML samples SU532, SU582, and SU480. H/E stained ossicles are marked by black rectangles. (**b**) Representative images of May-Gruenwald-Giemsa staining performed on FACS-purified $CD45^+/CD33^+$ leukemia blasts derived from engrafted humanized ossicle niches. At least two ossicles were analyzed per sample. Scale bars indicate 20μm (left) and 50μm (right). (**c**) AML blasts engrafted in humanized ossicle niches

recapitulate the subclonal architecture of the original blasts. Customized hybrid capture sequencing (targeting the 130 most frequently mutated genes in AML) of engrafted human AML samples SU306, SU480, SU532, and SU582. Genomic DNA (gDNA) was isolated form the original AML blasts prior to xenotransplantation (black diamonds), human AML blasts from directly transplanted humanized ossicle niches (red rectangles), non-transplanted ossicles (green rectangles), ossicle niches after intravenous transplantation (orange dots), and human AML blasts from mouse BM of engrafted mice (blue triangle). For samples SU532 and SU582 T-cells were used as germline control (cyan dots). Variant allele frequencies (y-axis) of detectable mutations (x-axis) are shown. Orange dotted line indicates the threshold of detection. Arrows mark samples with lower frequency (in non-Tx ossicle after iv Tx) or complete absence (in mouse BM after iv Tx) of *NPM1* mutation. (**d**) Humanized ossicle niches contain more absolute leukemia cells than corresponding total mouse BM. The absolute number of human leukemia cells within total mouse BM was determined as previously reported, based on counting $CD45.1^+$ total nucleated cells (TNC) in the femur of NSG mice and extrapolating.[4,5] Similarly engrafted ossicles were crushed and analyzed for CD45.1 TNC content. Total engrafted human leukemia cells were determined based on the percentage of human $CD45^+CD33^+$ leukemia engraftment. Bar graph shows mean total engrafted human leukemia cells (mean ± SD) in humanized ossicle niches (red bars) or corresponding mouse BM (grey bars) for primary AML samples SU306, SU480, SU532, SU582 and all samples combined, as indicated (left to right). Unpaired student's t-test was used for statistical analysis.

Supplementary Figure 6. Humanized ossicle niches facilitate robust engraftment of acute promyelocytic cells from primary samples SU589, SU490, SU718, and SU748 with APL leukemia-initiating cells likely residing within a lineage-committed progenitor, but not HSC or MPP. FACS analysis showing human engraftment at 6 – 8 and 12 – 24 weeks post-transplantation of 1×10^6 primary T cell-depleted MNC from primary APL samples SU490,

SU718, and SU748. Cells were directly transplanted into humanized ossicle niches, and transplanted ossicles (Tx ossicle), as well as mouse bone marrow (BM) were analyzed for the presence of human $CD45^+CD33^+$ myeloid and $CD45^+CD19^+$ lymphoid cells (left panels; N/A: not analyzed). May-Gruenwald-Giemsa (MGG) stained cytospins and interphase fluorescence in situ hybridization (FISH) for PML-RARA translocation on sorted human $CD45^+CD33^+$ blasts from APL-engrafted humanized ossicle niches are shown on the right. Yellow arrows mark chromosomal fusion events indicative for t(15;17). (**d**) FACS plots depicting pre-transplantation purity of FACS-purified bulk APL cells, HSC/MPP, and aberrant GMP (left panel) derived from APL sample SU490 and their engraftment kinetics within transplanted ossicles (right panel), demonstrating human $CD45^+$ cells further gated for myeloid cells ($CD45^+CD33^+$) and B lymphoid cells ($CD45^+CD19^+$). (**e**) PML-RARA FISH analysis of FACS-purified humanized ossicle niche-engrafted myeloid ($CD33^+$) and/or lymphoid ($CD19^+$) cells derived from the indicated transplanted populations 24 weeks post-transplantation of samples SU490 and SU748.

Supplementary Figure 7. Leukemia-initiating cells in myelofibrosis reside in the HSC compartment. Identical *JAK2*^{V617F} mutant allele burden in the engrafted cells and the primary sample prior to transplantation. (**a**) *JAK2* mutation status on FACS-purified hematopoietic stem and progenitor cells (black dots) as well as mature myeloid cells (monocytes: dark grey dots; granulocytes light grey dots) and (**c**) engrafted human $CD33^+$ myeloid cells derived from transplanted FACS-purified HSC (SU482) was tested by custom Taqman single nucleotide polymorphism (SNP) genotyping assays. FACS-purified cells were either classified as WT (*JAK2*^{WT}, y-axis) or mutated (*JAK2*^{V617F}, x-axis) based on comparison with cell lines know to be either WT (K562, red rectangles) or mutated (HEL, green rectangles), or no template control (NTC, blue rectangles). Reactions were performed in triplicates. (**b**) FACS plots depicting pre-transplantation purity (upper panel) of FACS-purified HSC, MPP, and CMP isolated from MF sample SU239.B (*CALR*^{mut}) and the percentage of $CD45^+$ engraftment within transplanted

ossicles eight weeks post-transplantation (lower panel) further gated for myeloid cells ($CD45^+CD33^+$) and B lymphoid cells ($CD45^+CD19^+$). (**d**) *CALR* mutation status in engrafted human $CD33^+$ myeloid cells derived from transplanted FACS-purified HSC (SU239.B) was determined by Sanger sequencing of exon 9. Sequencing chromatograms showing WT sequence with deletion marked by the red bar (upper panel) and 52 bp deletion at position c.1092_1143 (lower panel) in cells isolated from an engrafted ossicle transplanted with FACS-purified HSCs ($CD34^+CD38^-CD45RA^-CD90^+$)

Supplementary Table 1: Characteristics of primary AML samples

Supplementary Table 2: Characteristics of primary ALL samples

Supplementary Table 3: inv 16 FISH on primary AML samples and engrafted cells

Supplementary Table 4: Limiting dilution analysis and leukemia-initiating cell frequency for primary samples SU028 and SU048

Supplementary Table 5: PML-RARA FISH on primary APL subpopulations

Supplementary Table 6: Characteristics of myelofibrosis samples and transplanted subpopulations

Supplementary Table 7: Transplantation and engraftment outcomes of primary myelofibrosis samples

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Supplementary Table 1. Characteristics of primary AML samples

Sample ID	specimen type	disease status	WHO classification	FAB classification	cytogenetics	mutations/indels	% blasts
SU306	pB	de novo	AML NOS	M5a	n.d.	FLT3-TKD, NMP1, IDH2	55
SU480	pB	de novo	AML NOS	M1	46,XX	DNMT3A, FLT3-ITD, CEPBA	77
SU532	pB	de novo	AML NOS	n.d.	46,XY	FLT3-ITD, NPM1	94
SU582	pB	de novo	AML NOS	n.d.	46,XX	FLT3-ITD, NPM1, IDH1, NRAS	91
SU490	pB	de novo	APL with PML/RARA	M3	46,XY, t(15;17)(q24;q21)	FLT3-ITD	94
SU589	pB	de novo	APL with PML/RARA	M3	FISH: positive for PML-RARA	n.d.	93
SU718	BM	de novo	APL with PML/RARA	M3	46,XY,t(15;17)(q24;q21) FISH: positive for PML-RARA	FLT3-ITD, NRAS	89
SU748	pB	de novo	APL with PML/RARA	M3	46,XY,t(15;17)(q24;q21) FISH: positive for PML-RARA	FLT3-ITD	68
SU380	pB	de novo	AML with inv(16)(p13;q22) or t(16;16)(p13;q22)	n.d.	46,XY,inv(16)(p13q22)	NRAS, C-KIT	63
SU080	BM	de novo	AML with inv(16)(p13;q22) or t(16;16)(p13;q22)	M5	46,XY,inv(16)(p13q22)	none	60
SU430	pB	de novo	BAL	n.d.	46,XY, der(7)t(7;11)(q11.2;q13)	IDH1, DNMT3A	98
SU028	Leuk	relapsed	AML NOS	M5	complex karyotype	DNMT3A, TET2, ASXL1, NPM1, FLT3-ITD	95
SU048	Leuk	de novo	AML NOS	n.d.	n.d.	DNMT3A, TET2, NPM1, FLT3-ITD	94
SU507	pB	de novo	BAL	n.d.	complex karyotype	none	38
SU421	BM	de novo	AML with t(8;21)(q22;q22)	M2	46,XY,t(8;21)(q22;q22)	none	71

Abbreviations: pB: peripheral blood; Leuk: leukapheresis; FAB: French American British; NOS: not otherwise speficied; BAL: biphenotypic acute leukemia; n.d.: not determined

Supplementary Table 2. Characteristics of primary ALL samples

Sample ID	specimen type	disease status	WHO classification	cytogenetics	% blasts
SU410	pB	de novo	T-ALL	46,XY	92
SU079	Leuk	de novo	B-ALL	46,XY,t(9;22)(q34;q11.2)	96

Abbreviations: pB: peripheral blood; Leuk: leukapheresis; BM:

Supplementary Table 3. Inv16 FISH on primary AML samples and engrafted cells

SU080			
Population	**No. of nuclei analyzed**	**Results**	
Original blasts	200	Positive (81.5%)	nuc ish(CBFBx2)(5'CBFB sep 3'CBFBx1) [163/200]
Engrafted sorted blasts (CD45+/CD33+)	200	Positive (76%)	nuc ish(CBFBx2)(5'CBFB sep 3'CBFBx1) [151/200]
SU380			
Population	**No. of nuclei analyzed**	**results**	
Original blasts	200	Positive (90%)	nuc ish(CBFBx2)(5'CBFB sep 3'CBFBx1) [180/200]
Engrafted sorted blasts (CD45/CD33+)	200	Positive (84%)	nuc ish(CBFBx2)(5'CBFB sep 3'CBFBx1) [168/200]

Supplementary Table 4. Limiting dilution analysis and leukemia-initiating cell frequency for primary samples SU028 and SU048

SU028				SU048		
Tx route	Cell dose	Tested	Engrafted	Cell dose	Tested	Engrafted
Intraossicle Tx	1000	4	4	1000	4	4
	500	4	4	500	4	4
	100	8	8	100	8	1
	1	24	1			
Intrafemoral Tx	1000	2	2	1000	2	2
	500	2	1	500	2	0
	100	2	0	100	2	0
LIC frequency						
Tx route	Upper	Estimate	Lower	Upper	Estimate	Lower
Intraossicle Tx	6	**20**	68	125	**271**	588
Intrafemoral Tx	166	**540**	1765	388	**2669**	18375

Supplementary Table 5. PML-RARA FISH on primary APL subpopulations

SU191			
FACS sorted population	**No. of nuclei analyzed**	**results**	**comments**
HSC	42	negative	no cells with PML-RARA fusion observed
MPP	139	negative	no cells with PML-RARA fusion observed
CMP/MEP	133	negative	no cells with PML-RARA fusion observed
$CD34^{lo/-}$ aberrant GMP	200	positive	89% with PML/RARA fusion (178/200)

SU490			
FACS sorted population	**No. of nuclei analyzed**	**results**	**comments**
HSC	200	negative	0.05% with PML-RARA fusion (1/200)
MPP	200	negative	no cells with PML-RARA fusion observed
$CD34^{lo/-}$ aberrant GMP	200	positive	90% with PML/RARA fusion (180/200)

SU515			
FACS sorted population	**No. of nuclei analyzed**	**results**	**comments**
HSC/MPP	143	negative	no cells with PML-RARA fusion observed
CMP	180	negative	no cells with PML-RARA fusion observed
$CD34^{lo/-}$ aberrant GMP	200	positive	7.5% with PML/RARA fusion (15/200)

SU648			
FACS sorted population	**No. of nuclei analyzed**	**results**	**comments**
HSC	141	negative	no cells with PML-RARA fusion observed
MPP	162	negative	no cells with PML-RARA fusion observed
CMP/MEP	200	negative	no cells with PML-RARA fusion observed
$CD34^{lo/-}$ aberrant GMP	200	positive	90% with PML/RARA fusion (180/200)

Supplementary Table 6. Characteristics of myelofibrosis samples and transplanted subpopulations

Sample ID	Specimen type	WHO classification	JAK2 V617F mutation status (% positive cells)
SU094.D	pB	PMF	92
SU180	pB	PMF	56
SU415	pB	PMF	95
SU482	pB	Secondary MF (post-ET)	81
SU239.B	pB	Secondary MF (post-ET)	CALR mutation positive

Abbreviations: pB: peripheral blood; PMF: Primary Myelofibrosis; ET: Essential Thrombocythemia: CALR: calreticulin

Sample ID	Transplanted Population	No. of transplanted cells (per ossicle)
SU482	$CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$ (HSC)	30,000
	$CD34^{+}CD38^{-}CD90^{-}CD45RA^{-}$ (MPP)	13,000
	$CD34^{+}CD38^{+}CD123^{+}CD45RA^{-}$ (CMP)	170,000
SU239.B	$CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$ (HSC)	3,700
	$CD34^{+}CD38^{-}CD90^{-}CD45RA^{-}$ (MPP)	2,300
	$CD34^{+}CD38^{+}CD123^{+}CD45RA^{-}$ (CMP)	6,400

Abbreviations: HSC: hematopoietic stem cells; MPP: multipotent progenitor cells; CMP: common myeloid progenitor cells

Supplementary Table 7. Transplantation and engraftment outcome of primary myelofibrosis samples

Sample ID	Transplanted population	Number of cells (transplanted per ossicle)	Engraftment (engrafted ossicles/total ossicles)
SU094.D	$CD34^+CD38^-$	2,000; 8,000	3/3; 4/4
	$CD34^+CD38^+$	12,500	0/4
SU180	$CD34^+$	50,000	4/4
SU415	$CD34^+CD38^+$	8,000; 4,000	2/2; 2/2
SU482	$CD34^+CD38^+$	30,000	4/4
	$CD34^+CD38^+$	150,000	0/4